App. No. 10/521,234
Office Action Dated July 22, 2009

REMARKS

Favorable reconsideration is respectfully requested in view of the above amendments and following remarks. Claim 15 has been amended editorially. No new matter has been added. Claims 11, 14 and 15 are pending.

Information Disclosure Statement

A line was crossed through the Stennicke et al. reference (Stennicke and Salvesen, *The Journal of Biological Chemistry*, Vol. 272, pp. 2517-23 (1997)) by the Examiner. However, Applicants respectfully submit that this reference was filed on February 3, 2009 in conformity with MPEP 606. Therefore, Applicants request consideration of this reference. For the Examiner's convenience, a copy of this reference is included herewith.

Claim Objections

Claim 15 is objected to because of informalities. Claim 15 has been amended, taking the issues noted in the objection into account. Withdrawal of the objection is requested.

Claim rejections - 35 U.S.C. § 103

Claims 11, 14 and 15 are rejected under 35 USC 103(a) as being unpatentable over Komori et al. (EP 1002874) and Glossary of class names of organic compounds (PAC, 1995, 67, 1307, pages 1351-1396), in view of Bauman et al. (US Patent No. 4,265,810) and Ledis et al. (US Patent No. 5,731,206) and further in view of Kaminagayoshi et al. (EP 0158964) and Ishimaru et al. (US Patent No. 6,127,138). Applicants respectfully traverse the rejection.

The rejection contends that the tetrazolium compound of Komori could be considered as both a nitro and a sulfonic compound. The rejection then contends that it would have been obvious to replace the nitro and sulfonic compounds as taught by Komori with those of Bauman and Ledig. Applicants respectfully submit that the rejection is relying on the improper use of hindsight in the interpretation of the reference.

In particular, the references are not faced with the same or similar problems. Komori aims to eliminate the external influence of reducing substances in measuring the amount of glycated proteins, particularly glycated hemoblogin in erythrocytes. Ledis aims to differentiate leukocytes while keeping them intact. Bauman relates to synthesizing a

dye from a dye intermediate. A person of ordinary skill in the art of measuring glycated proteins in blood, as taught by Komori, would not turn to the dye intermediate of Bauman or the leukocyte differentiating reagents of Ledis.

The rejection further contends that a person of ordinary skill in the art would have been motivated to modify the method as taught by Komori by using 4-aminoazobenzene-4'-sulfonic acid sodium salt of Bauman and 2,4-dinitrophenol as taught by Ledis with a reasonable expectation of success in order to provide a method of measuring the amount of a glycated protein. However, the references fail to provide any reason to expect that replacing Komori's tetrazolium compound with a nitro or sulfonic compound of Bauman and Ledis that is not a tetrazolium compound would not disrupt the measurement of the hydrogen peroxide.

In particular, it can be clearly understood from Komori as a whole that this reference teaches the use of a compound with a particular ring structure, namely the following:

to achieve a particular effect, namely eliminating the influence of any reducing substance when measuring the amount of hydrogen peroxide, which is highly susceptible to external factors. Komori fails to provide any guidance or any experimental data to show that replacing a compound having the above ring structure with a nitro or sulfonic compound that does not include the above ring structure would not disrupt the measurement of the hydrogen peroxide, let alone achieve the same effects as that of a compound having the above ring structure.

Ledis, Bauman, Ishimaru and Kaminagayoshi do not remedy the deficiencies of Komori. Ledis is focused on the use of a lytic reagent that rapidly partitions whole blood sample into an essentially intact leukocyte fraction and a lysed erythrocyte fraction, and is silent as to the effect of the lytic reagent on the measurement of hydrogen peroxide. Bauman teaches the conversion of a dye intermediate to a dye. Ishimaru discloses the use of a metalloproteinase. However, Bauman and Ishimaru are likewise silent as to effects of

a nitro or sulfonic compound that does not include the above ring structure on the measurement of hydrogen peroxide. Kaminagayoshi discloses alkylbenzene sulfonates, which do not correspond to any of the claimed compounds. Accordingly, even when combined, the references fail to meet the features of claim 15. Accordingly, claim 15 and its dependent claims are patentable over the references, taken alone or together.

In view of the above, favorable reconsideration in the form of a notice of allowance is requested. Any questions or concerns regarding this communication can be directed to the attorney-of-record, Douglas P. Mueller, Reg. No. 30,300, at (612) 455.3804.

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Biochemical Characteristics of Caspases-3, -6, -7, and -8*

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The observation that the nematode cell death effector gene product Ced-3 is homologous to human interleukin-1β-converting enzyme (caspase-1) has led to the discovery of at least nine other human caspases, many of which are implicated as mediators of apoptosis. Significant interest has been given to aspects of the cell biology and substrate specificity of this family of proteases; however, quantitative descriptions of their biochemical characteristics have lagged behind. We describe the influence of a number of environmental parameters, including pH, ionic strength, detergent, and specific ion concentrations, on the activity and stability of four caspases involved in death receptor-mediated apoptosis. Based on these observations, we recommend the following buffer as optimal for investigation of their characteristics *in vitro*: 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2. Caspase activity is not affected by concentrations of Ca^{2+} below 100 mM, but is abolished by Zn^{2+} in the submicromolar range, a common characteristic of cysteine proteases. Optimal pH values vary from 6.8 for caspase-8 to 7.4 for caspase-3, and activity of all is relatively stable between 0 and 150 mM NaCl. Consequently, changes in the physiologic pH and ionic strength would not significantly alter the activity of the enzymes, inasmuch as all four caspases are optimally active within the range of these parameters found in the cytosol of living and dying human cells.

Apoptotic cell death is a process that enables metazoans to eliminate cells that are damaged, mislocated, or have become superfluous, and is characterized by controlled proteolysis of cellular components resulting from activation of an in-built program (2, 3). The signal for the execution of the cell may come from various stimuli: specific death receptor ligation (4), ionizing radiation (5), anti-neoplastic drugs (6), and growth factor withdrawal (7). However, despite the variety of death signals, the key features of execution appear to be quite similar; the death signal converges upon the activation of a number of proteases, which in turn cleave protein substrates (8, 9), thus giving rise to characteristic apoptotic morphology.

Since the discovery that *ced-3*, a key effector gene of programmed cell death in *Caenorhabditis elegans*, exhibited homology with interleukin 1β-converting enzyme (ICE[1] or caspase-1), the involvement of proteolytic enzymes in apoptosis has been an issue of significant interest (10–12). This has resulted in the cloning of several mammalian genes encoding ICE/Ced-3 homologues, known commonly as caspases (13), several of which are important for promotion of the death pathway in mammals (reviewed in Ref. 9). However, with the notable exception of caspase-1 (14–16), little attention has been given to the key biochemical properties of these enzymes, which is important for understanding the effect of the intracellular environment on their activity. For example, changes in pH, redox potential, and Zn^{2+} concentration all have effects on apoptosis (17–21). In the present article, we present a characterization of some of the basic biochemical properties of four of the caspases. We have chosen to focus on those that play a central role in the apoptotic pathway initiated by ligation of the death receptors Fas and tumor necrosis receptor 1: caspase-3 (Yama/CPP32/apopain), caspase-6 (Mch2), caspase-7 (Lap3/Mch3/CMH1), and caspase-8 (FLICE/MACH) (22–25).

EXPERIMENTAL PROCEDURES

Materials—Active caspases-3, -6, -7, and -8 were expressed in *Escherichia coli* and isolated as described previously (22, 24, 27). The expression constructs for caspases-3, -6, and -7 contained a His_6 tag at the C terminus of the full-length protein, while caspase-8 was constructed to have a His_6 tag at the N terminus replacing residues 1–216 of the zymogen. The concentrations of the purified enzymes were determined from the absorbance at 280 nm based on the molar absorption coefficients for the caspases calculated from the Edelhoch relationship (28): caspase-3 (ϵ_{280} = 28000 M^{-1} cm^{-1}), caspase-6 (ϵ_{280} = 28000 M^{-1} cm^{-1}), caspase-7 (ϵ_{280} = 24510 M^{-1} cm^{-1}), and caspase-8 (ϵ_{280} = 27390 M^{-1} cm^{-1}). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC) was purchased from Enzyme System Products. DTT was from Diagnostic Chemicals Limited. Sucrose was from Mallinckrodt. All other chemicals were from Sigma. Z-DEVD-fluoromethyl ketone was the kind gift of Joe Krebbs, IDUN Pharmaceuticals.

Determination of the pH Dependence of the Caspases—The pH dependence of the hydrolysis of the substrate Z-DEVD-AFC were evaluated in the pH range 5.5–10. The enzymatic reaction was carried out at 37 °C in the following buffers: 20 mM MES (pH 5.5–6.5), 20 mM HEPES (pH 6.2–7.2), 20 mM PIPES (pH 6.9–8.1), 20 mM Bicine (pH 7.8–9.0) or 20 mM CHES (pH 8.8–10.0), containing NaCl, DTT (fresh), EDTA, CHAPS, and sucrose at optimized concentrations as described under "Results and Discussion." For reasons discussed later, the optimal buffer used as a basis for further studies was 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2. The enzyme concentrations used were 1.2 nM (caspase-3), 15 nM (caspase-6), 3 nM (caspase-7), and 80 nM (caspase-8). The initial rates of enzymatic hydrolysis were measured by release of AFC from the substrate Z-DEVD-AFC (0.1 mM) as emission at 505 nm upon excitation at 400 nm using a Perkin-Elmer LS50B fluorimeter equipped with a thermostated plate reader. The pH dependences of the initial rates of hydrolysis for all four caspases were fitted to a bell shape described for two ionizing groups by the equation $v = (\text{limit} \times \log(pH - pK_{a1})/(\log(2 \times pH - pK_{a1} - pK_{a2}) + \log(pH - pK_{a1}) + 1))$ using Grafit 3.01 (29).

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[1] The abbreviations used are: ICE, interleukin-1β-converting enzyme; Z, carbobenzoxy; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; AFC, 7-amino-4-trifluoromethyl coumarin; PAGE, polyacrylamide gel electrophoresis.

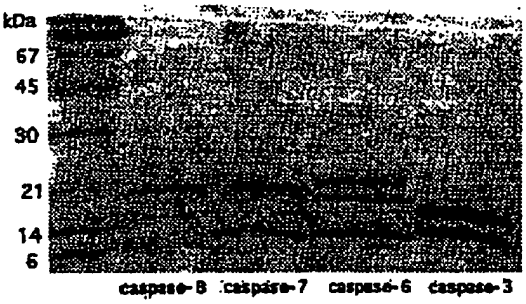

FIG. 1. SDS-PAGE analysis of the purified recombinant caspases. Approximately 5 μg of each caspase was electrophoresed in a linear 5–15% SDS-PAGE gel (40), followed by staining with Coomassie Blue. The gel demonstrates the presence of the large and small subunits characteristic of the activated enzymes, as well as the purity of the preparations used in this study.

Determination of the Zn^{2+} and Ca^{2+} Ion Sensitivity of the Caspases—The sensitivity toward Zn^{2+} and Ca^{2+} was determined in optimal buffer (without EDTA), containing varying concentrations of $ZnCl_2$ or $CaCl_2$ as described above. A concentration of 20 mM β-mercaptoethanol was used to replace DTT because it does not chelate zinc to the same extent as DTT. The influence of the concentration of the reductant on the zinc sensitivity was exploited for caspase-3 using varying concentrations of β-mercaptoethanol. The inhibition of the caspases by Zn^{2+} was fitted to an equation describing simple competitive inhibition, $v = V_{max}/(1 + ([K_i/[S]) \times (1 + ([Zn^{2+}]/K_{ic}))$ using Grafit 3.01 (29).

Determination of the Sensitivity of the Caspases to Ionic Strength—The sensitivity toward ionic strength was determined in optimal buffer containing varying concentrations of NaCl as described above.

Determination of the In Vitro Stability of the Caspases—The stability of the four caspases was determined by incubating the enzymes in optimal buffer at 0 °C or 37 °C. At various time points, a sample was withdrawn and the activity was determined as described above.

RESULTS AND DISCUSSION

The Caspases—Heterologous expression of the caspases is required to obtain sufficient amounts of starting material for a rigorous characterization. Although the mechanism is not understood, when expressed in *E. coli*, these caspases spontaneously undergo what appears to be autoprocessing to yield the appropriate subunits characteristic of the active enzymes (Fig. 1). Processing at interdomain Asp residues was confirmed for all of the recombinant proteases by sequencing of the N termini of the two subunits (see Refs. 22 and 27 for further details). Note that both the large and small subunits migrate as homogeneous bands in SDS-PAGE, with the exception of the large subunit of caspase-8, which, based on N-terminal sequencing, represents alternative cleavages at the C terminus of the large subunit. The N-terminal peptides of caspases-3, -8, and -7 were also removed during processing, as demonstrated to occur during Fas-mediated apoptosis *in vivo* (22). Caspase-8 could not be expressed as a full-length protein and thus was engineered with a 21-residue linker (24, 27) that replaces the 216-residue N-terminal segment removed during its activation *in vivo* after Fas ligation (25, 26). Consequently, with the exception of the terminal purification tags, the proteins are essentially identical to the forms identified or expected *in vivo* in apoptotic cells. On the basis of titration with the active site-directed caspase inhibitor Z-DEVD-fluoromethyl ketone, all proteases were 100% active, based on absorbance at 280 nm, with the exception of caspase-8, which was 50% active.

Effects of Sucrose and Detergent—Common to all the caspases is a distinct preference for aspartic acid in the P_1 position.[3] In the present study, we have used Z-DEVD-AFC as the test substrate even though the four caspases investigated exhibit some degree of P_4 preference. Use of this substrate is suitable, inasmuch as we are interested in the properties of the caspases and not in the possible effects that the change of various parameters may have on the protein substrates.

TABLE I
Effect of removing various compounds from the assay buffer
The effects shown describe the fractional activity in buffer without components relative to one containing them.

	Caspase-3	Caspase-6	Caspase-7	Caspase-8
CHAPS	0.59	0.03	0.39	0.30
Sucrose	0.95	0.82	0.81	0.92
NaCl	0.97	1.69	0.78	0.97

Initially, the requirements for various components were investigated in a buffer based on that used by Thornberry *et al.* for caspase-1 (11): 20 mM HEPES, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS, 10% sucrose, pH 7.4. Table I demonstrates the effects of removal of some of these component from the assay buffer. All four caspases lose over 40% of their activity upon removal of CHAPS from the buffer, and the effect is more dramatic with caspase-6 than with caspases-3, -7, and -8. Only minor beneficial effects are found with sucrose and NaCl; in the case of caspase-6, there is a significant reduction in activity in the presence of NaCl, which will be discussed in detail below. However, 100 mM NaCl is required in the assay buffer to maintain a consistent ionic strength when varying pH. A relatively high concentration of DTT (10 mM) is required for full activity of the recombinant enzymes. They may be preactivated by DTT and the DTT removed by gel filtration; however, if neither reducing agent nor EDTA is present in the exchange buffer, the activity declines rapidly, presumably due to oxidation of the catalytic cysteine (data not shown). EDTA (1 mM) is incorporated into the assay buffer to avoid inactivation by trace metals.

Effects of pH—Only minor differences were observed in the pH profiles of the four caspases. The bell-shaped pH dependence signifies the existence of one active form of the enzyme with the increase in activity most likely due to the de-protonation of the catalytic Cys residue. In this respect, the caspases closely resemble other unrelated cysteine proteases in their activity pH profiles (30). Caspase-3 was found to be active over a broader pH range with an optimum slightly higher than the other three (see Fig. 2). Although we have analyzed the pH dependence of all four enzymes as a simple bell-shaped curve, there is a faster than expected drop-off in activity at low pH, most clearly observed with caspases-3 and -6. This indicates that more than one group is protonating, possibly another group on the enzyme, or the substrate carboxylate(s), inasmuch as the three-dimensional structure of caspases-1 and -3 demonstrates binding of unprotonated side-chains in its specificity pockets (31–33). The pH dependence of these caspases also indicates that they all are fully active within the pH range found in normal as well as apoptotic cells, designated by the *shaded background* in Fig. 2 (18, 21). It is possible that changes in pH during apoptosis may affect caspase activity indirectly by altering the structure of a particular set of natural substrates. However, this hypothetical event would change only the susceptibility of the substrate, not the activity of the caspases.

Effects of Ionic Strength—We used NaCl in the range 0–1 M in assay buffer to address the dependance of ionic strength on caspase activity. Differential effects were found depending on the enzyme, with caspases-3 and -6 having fairly flat profiles,

[3] Binding site nomenclature is in accordance with the nomenclature of Schechter and Berger (1).

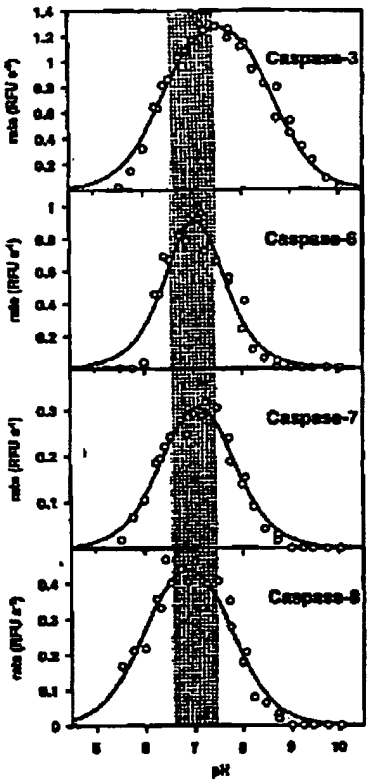

FIG. 2. The pH dependence of the four caspases for the hydrolysis of the synthetic peptide substrate Z-DEVD-AFC. The dependence was fitted to a bell shape characterized by the following pK_a values: pK_{a_1} = 6.4 and pK_{a_2} = 8.6 for caspase-3, pK_{a_1} = 6.9 and pK_{a_2} = 7.2 for caspase-6, pK_{a_1} = 6.8 and pK_{a_2} = 7.7 for caspase-7, and pK_{a_1} = 6.0 and pK_{a_2} = 7.7 for caspase-8. The *shaded area* illustrates the pH range found in normal and apoptotic cells, with the latter favoring lower pH (18, 21).

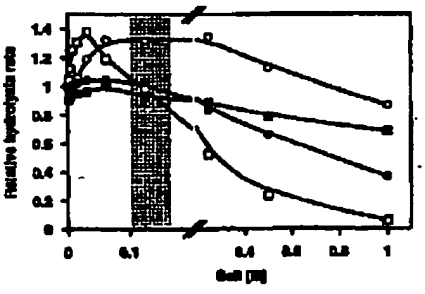

FIG. 3. NaCl dependence of caspases. Caspase-3 (○), -6 (□), -7 (▽), and -8 (◇) were incubated under optimal buffer conditions, with the indicated concentration of NaCl, and initial rates of substrate hydrolysis determined. The rates of hydrolysis have been normalized to the rate of hydrolysis in the absence of NaCl. The *shaded area* illustrates the range of ionic strength normally found in the cytosol (34).

whereas caspases-6 and -7 demonstrated maximal activity at 0.03 M and 0.25 M (Fig. 3). Although the activity of caspase-6 declined faster than the others as ionic strength increased, none of the enzymes demonstrated substantial adverse effects

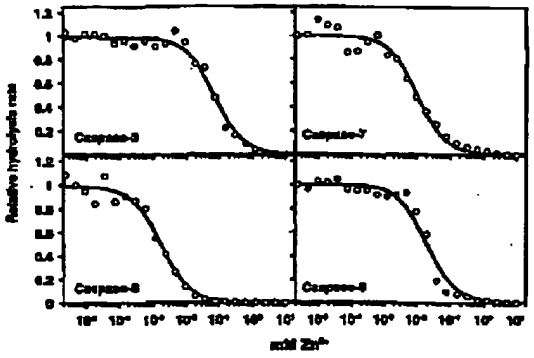

FIG. 4. Sensitivity of the four caspases to the presence of Zn^{2+}. Caspases were incubated under optimal buffer conditions, with DTT replaced by β-mercaptoethanol, at the indicated concentration of Zn^{2+}, and initial rates of substrate hydrolysis determined. The apparent binding constants for Zn^{2+} to the individual caspases ($K_{D_{app}}$) are 8.5 μM for caspase-3, 0.3 μM for caspase-6, 1.7 μM for caspase-7, and 1.9 μM for caspase-8.

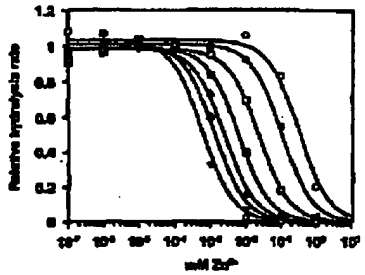

FIG. 5. Influence of the concentration of β-mercaptoethanol on the apparent binding constant for Zn^{2+} to caspase-3. The influence of β-mercaptoethanol on $K_{D_{app}}$ were investigated using the concentrations 0.25 mM (▽), 0.5 mM (▽), 1 mM (△), 2 mM (△), 4 mM (■), 8 mM (□), 16 mM (●), and 32 mM (○).

on their activity in the physiologic range of ionic strength (34), designated by the *shaded background* in Fig. 3. The apparent stability to substantial changes in ionic strength indicates that this would not be limiting during commitment to apoptosis.

Effects of Zn^{2+} and Ca^{2+}—Several studies have reported that Zn^{2+} inhibits apoptosis. Originally, this effect was believed to be due to the inhibition of nucleases; however, caspase-6 (17) and, more recently, caspase-3 (20) have been found to be inhibited completely by 2 mM Zn^{2+}. The influence of transition metal ions on the activity of cysteine proteases has been well established for a long time; for instance, members of the papain family are sensitive to Zn^{2+}, mercury, and various organomercurials (35, 36). Because DTT chelates Zn^{2+}, we compared caspases for sensitivity to this ion in the presence of 20 mM β-mercaptoethanol, which we determined to be the concentration of this reductant required for optimal activity of the recombinant enzymes (data not shown). Due to the inherent tendency of Zn^{2+} to react with thiols, we can only obtain an apparent binding constant and, under these conditions, all the caspases are inhibited by small amounts of Zn^{2+}, although there are significant differences in the affinity (Fig. 4). Caspase-6 is most readily inhibited by Zn^{2+}, completely inactivated by 0.1 mM, and caspase-3 is the least sensitive, requiring more than 1 mM for complete inactivation. To estimate the real binding affinity, we probed the influence of reductant on

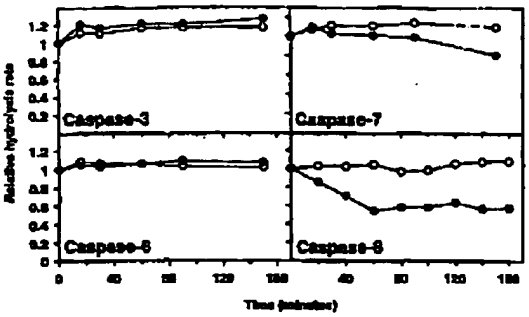

FIG. 6. *In vitro* stability of the caspases. The stability of the caspases in optimal buffer at 0 °C (○) and 37 °C (●) were determined by measuring the residual activity as a function of time.

the inhibition of caspase-3 by Zn^{2+}. Not surprisingly, there was a significant influence of the concentration of β-mercaptoethanol on the $K_{Zn_2(E)}$ giving rise to values converging on an approximate value of 0.15 μM (Fig. 5). From these results, it is quite evident that Zn^{2+} is a good inhibitor of the caspases, albeit very dependent on the thiol content, and therefore presumably the redox potential of the cell. The influence of Ca^{2+} was investigated in a similar manner and was found to have no effect on the activity of any of the caspases at concentrations up to 100 mM (data not shown). Thus, the reported role of Ca^{2+} in apoptosis (see, for example, Ref. 37) is unlikely to be due to any effect on the caspases.

In Vitro Stability—On the basis of the foregoing results, the optimal general caspase buffer was designated as 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2. The stability of the four caspases was tested by incubating the enzymes at 0 °C or 37 °C in the optimal assay buffer and determining the activity at various times (Fig. 6). None of the caspases showed any decrease in activity at 0 °C over the 150-min period. Caspases-3 and -6 retained full activity for 150 min at 37 °C, whereas caspases-7 and -8 showed an appreciable decrease in activity. To verify that the decrease in activity observed at 37 °C with caspases-7 and -8 was not due to sample variation, the experiment was performed with two different preparations of these enzymes giving rise to almost identical results, reducing the probability that the decrease in activity is associated with sample variations. The reason for the decrease in activity is not clear; however, SDS-PAGE analysis of caspases incubated at 0 °C and 37 °C for 150 min does not reveal any indications of degradation (data not shown). Based on these observations, the most probable explanation is a conformational change, possibly due to slow dissociation of the subunits after dilution into assay buffer, as originally described for caspase-1 (11). This assumption is supported because the decrease in activity observed with caspase-8 appears to approach a level of approximately 50%, and remains there for an extended period of time. Whether such dissociation occurs in a cell under physiologic conditions remains an open question, but it is evident that none of the investigated caspases undergo autolysis that will significantly affect their role in apoptosis. This is in contrast to caspase-1, which has been shown to inactivate spontaneously by autolytic degradation of its small subunit (38).

Biologic Perspective—The results demonstrate that all four caspases are optimally active under normal physiologic conditions. We have to activate the recombinant enzymes by adding thiols, presumably because of reversible modification of the catalytic cysteine during expression and purification. *In vivo*, however, the glutathione balance would favor the reduced form, with the result that, once processed from their single chain zymogens, the caspases would be fully active. We do not rule out the possibility that natural caspase substrates are affected by changes in environmental parameters that would alter their susceptibility to specific proteolysis *in vivo*. In this context, caspase-1 was shown to exhibit a marked salt dependence due to effects of NaCl on the substrate pro-interleukin-1β, but not on a synthetic peptidyl substrate (39). However, changes in the pH and ionic strength of the cytosol would not significantly alter the activity of the enzymes themselves, inasmuch as all four caspases are optimally active within the range of these parameters found within cell cytosols, irrespective of their metabolic status.

Acknowledgments—We thank Yuri Lazebnik for helpful discussion of this manuscript, Qiao Zhou for performing the active site titration of the caspases, and Annamaria Price and Scott Snipas for technical assistance. We thank Joe Krebbs for providing us with Z-DEVD-fluoromethyl ketone.

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